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ORIGINAL PAPER

Shade induced changes in biomechanical petiole properties in the stoloniferous herb *Trifolium repens*

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Abstract Increased cell number and cell length both contribute to shade induced elongation of petioles which enables stoloniferous plants to place their leaf lamina higher up in the canopy. Although petiole elongation is assumed to be beneficial, it may also imply costs in terms of decreased biomechanical stability. We test the hypothesis that shade induced elongation changes the biomechanical properties of petioles and that the underlying mechanisms, cell division and cell elongation, differentially affect biomechanical properties. This was done by subjecting 14 genotypes differing in the relative contribution of cell size and cell number to shade induced elongation responses to high light conditions and to simulated canopy shade. Developmental traits (cell size and cell number), morphological traits characterizing the petioles, as well as biomechanical characteristics were measured. Our results show that, comparable to stems of non-clonal plants, the rigidity of a petiole's tissue (the Young's modulus) increases, leading to increased flexural stiffness of petioles subjected to shading. Increased flexural stiffness proved to be associated with increased performance under shaded conditions. Our results also indicate that cell number affected the material properties and the flexural stiffness of petioles. However, the degree and pattern of the effects differed between light environments. Shade induced increase in cell number translated into shade induced increase of Young's modulus and flexural stiffness. Genotypes producing relatively larger cells under shaded conditions experienced a decrease in tissue rigidity. In concert our results indicate that the pattern of selection on flexural stiffness, and thereby also on shade induced changes of cell number and cell size differs among light environments.

Keywords Cell number · Cell size · Flexural stiffness · Petiole elongation · Shade induced plasticity · Stoloniferous plants · Young's modulus

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Introduction

Most natural environments are characterized by fine-grained temporal and spatial variation in the availability of essential resources such as light, water and nutrients thereby exerting different selection pressures on plant development and morphology (Kalisz 1986; Stewart and Schoen 1987; Stratton 1995; Stratton and Bennington 1996). If changes in phenotype and/or developmental pattern confer a fitness advantage adaptive plasticity will evolve (Dudley and Schmitt 1996; Kingsolver 1995). The photomorphogenetic induction of shade-avoidance responses in crowded plants is a well-studied example of adaptive plasticity (Ballaré et al. 1990; Casal and Smith 1989; Griffith and Sultan 2006; Morgan and Smith 1979; Schmitt et al. 2003; Schmitt and Wulff 1993). Upon shading many herbaceous plants elongate their vertically oriented spacers (i.e. internodes and/or leaf petioles) in order to place their leaves in higher positions of the canopy which results in improved light acquisition (Callaway et al. 2003; Dudley and Schmitt 1996; Franklin and Whitelam 2005; Huber et al. 1998; McGuire and Agrawal 2005; Schmitt and Wulff 1993; Tsukaya et al. 2002). Plastic spacer elongation has been shown to confer clear advantages in herbaceous canopies and can hence be expected to be under positive selection in a wide range of plant communities (Causin and Wulff 2003; Donohue et al. 2000; Huber and Wiggerman 1997; Leeflang et al. 1998; van Kleunen and Fischer 2003; Weinig 2000).

Shade induced spacer elongation is associated with other structural changes of the elongating organs. Elongation of internodes and petioles usually implies a change in resource allocation, leading to changed root:shoot ratio of shaded plants (Huber et al. 2004; Maliakal et al. 1999), reduced investment into defense (Cipollini 2004; Thaler and Bostock 2004) or to the production of longer, but thinner stems or stem analogous (Anten et al. 2005; Liu et al. 2007). Although the production of thinner stems may reduce cost in terms of the amount of carbohydrates used per unit of stem length, it may also entail significant costs in terms of reduced biomechanical stability, which carries the risk of lodging or breaking of the elongating structures (Anten et al. 2005; Henry and Thomas 2002; Mitchell 2003). However, plants in the shade tend to increase the rigidity of stem tissue (the Young's modulus, E) (Liu et al. 2007; Anten et al. 2005). The Young's modulus and the cross sectional area of the stem interact in determining the flexural stiffness of an organ, which describes how easily an organ of given length bends and is thus strongly related to its ability to carry it's own weight, and resist external forces such as wind (Niklas 1992; Read and Stokes 2006). An increase in the Young's modulus can therefore at least in part compensate for a reduction in diameter.

Erect and clonal plants use two distinctively different types of spacers to shift their leaf blades higher up in the canopy. While in erect plants internodes are the main organ showing adaptive elongation responses, in clonal plants the vertically oriented petioles are assuming the same function (Huber 1996; Huber et al. 1998). From a biomechanical perspective, elongation of internodes and petioles are subjected to different constraints (Liu et al. 2007). In erect plants each internode has to support its own weight, as well as the weight of the internodes, branches and leaves that are formed above it. The extension of a given internode thus affects the positioning of leaves and branches situated on all successive internodes. In stoloniferous plants each leaf is supported by a separate petiole, which in terms of biomass use for vertical support is less efficient than producing a single stem; each petiole has to carry its weight in addition to that of the lamina. Extension of petioles, in addition, only affects the lamina placement of a single module, but not of other attached modules. The modular structure of stoloniferous plants and the potential of each

module to adjust its own structure to the prevalent environmental conditions make clonal plants very flexible and able to efficiently respond to fine scale variation in light conditions (de Kroon et al. 2005). Yet the biomechanics of stoloniferous plants have hardly been investigated and little is know about the consequences of shade induced petiole elongation for mechanical stability (Liu et al. 2007). Most studies on shade effects on the biomechanics of petioles have been conducted on trees (Niinemets and Fleck 2002; Niinemets and Valladares 2004).

Extension of plant structures can be achieved by either cell extension or cell division (Beemster et al. 2006). These two are distinctly different developmental processes which are separated in time and different genes are independently involved in the processes regulating cell proliferation or cell elongation (Tsukaya et al. 2002). As cell division involves additional investment into cell material, a spacer elongation through cell division may be more costly in terms of biomass compared to spacer elongation by means of cell elongation. On the other hand, tissue made of more but smaller cells might have a higher density of cell walls providing rigidity and strength, and thus be more resistant to buckling or rupture. Previous research has shown genetic variation in the relative contribution of both processes to shade induced elongation (Weijschede et al. submitted). It is, unclear however, in how far cell size and cell elongation affect biomechanical characteristics of petioles.

In this paper we test the hypothesis that the investment into the production of more, but shorter cells increases the biomechanical stability of petioles. We expect that petioles elongating primarily by increased cell number will be more rigid, and less likely to buckle, than those elongating primarily through (cheaper) cell elongation. This difference will affect the degree to which petioles can independently maintain their vertical position. Such different consequences associated with the relative contribution of cell size and cell number to shade induced petiole extension entails that different developmental processes will be selected for, depending on the specific environmental conditions and local structure of the vegetation.

We will present data on how shade-induced changes in developmental and morphological traits affect biomechanical characteristics of the petioles in the stoloniferous herb *Trifolium repens*. We aim at providing answers to the following research questions:

1. Do the biomechanical properties of petioles depend on light conditions?
2. Are the biomechanical properties of petioles linked to morphological traits such as petiole length, petiole thickness, leaf area and leaf weight?
3. Do the biomechanical properties of petioles depend on cell length and cell number per unit of petiole length?
4. Do shade-induced changes of cell number and cell length affect the biomechanical properties of petioles?
5. Do biomechanical properties of petioles affect plant performance?

Material and methods

Species description and pre-treatment conditions

Trifolium repens L. is an abundant species growing in pastures and lawns, on riverbanks and road-side verges throughout temperate Europe and other parts of the world. It produces

monopodial above-ground stolons which root on their nodes and form ramets consisting of an internode, a node with one leaf and an axillary meristem, and a root system. The axillary meristem can give rise to either a lateral stolon or an inflorescence (Huber and During 2000). Plants can produce two to three ramets (i.e. repeated modules) on the primary stolon per week. Petioles are the main structures determining the positioning of light acquiring structures in the canopy (Huber et al. 1998; Huber and Wiggerman 1997). Genotypes of this species vary greatly in petiole traits, such as constitutive petiole length (Weijschede et al. 2006), plastic petiole elongation (Weijschede et al. 2006), and in the extent to which cell division and cell elongation contribute to shade induced petiole elongation (Weijschede et al. submitted).

All plants used in this experiment were randomly collected in a floodplain pasture along the river Waal near Ewijk (the Netherlands, 51°52′54″N, 5°45′00″E) in 2001. The distance between sampled plants was at least 5 m. The genetic uniqueness of sampled plants was confirmed by molecular fingerprinting (AFLP, four primer combinations, 145 markers). After collection the plants were maintained under uniform outside conditions in the Botanical Garden of the Radboud University in Nijmegen. Plants were grown in individual pots in a substrate consisting of a 1:1 mixture of sand and potting compost. Plants were repotted twice a year. In autumn 2005, 14 genotypes were moved to the heated greenhouse and planted in flat trays filled with a 1:1 mixture of sand and potting compost. These 14 genotypes represent a subset of the 34 genotypes used by (Weijschede et al. 2006; Weijschede et al. submitted). Genotypes were chosen to represent a wide range of shade induced changes of petiole cell size and cell number and thus to represent the whole variation in developmental mechanisms regulating petiole extension reported by (Weijschede et al. submitted).

Treatments

In March 2006, two lateral cuttings were made from all genotypes, and these cuttings were planted individually into flat trays (l*b*h:16*14*4), filled with a 1:2 mixture of sand and sieved potting compost and an addition of slow release fertilizer (Osmocote+, Sierra International, 4 g l^{-1}) to prevent nutrient limitation. Each lateral cutting consisted of a rooted ramet and a lateral stolon consisting of 3–5 ramets. The cuttings were pinned to the soil with plastic coated wire to ensure good contact with the ground. The plants were covered with a transparent plastic foil for 3 days to reduce evaporation and minimize negative effects of planting on stolon development. The substrate was kept moist by watering three times a week. This planting was repeated in four temporal plots, with 3–4 days intermittent individual plantings. The total number of plants used in the experiment was 112 (14 genotypes, two treatments, four blocks).

About 4 weeks after planting the plants were subjected to the shading treatments. Shading was induced by placing the plants into shade cages covered by one layer of a green plastic film (Lee filter no 122, fern green, Lee Colortran International, Andover, UK), which reduced light availability to 31%, and the red:far-red ration to 0.25. Control plants were grown in cages covered with a transparent plastic (Lee filter no 130, clear, light transmittance of 76%, red:far-red ratio:1.51) to keep microclimatic conditions comparable between shading treatments (de Brouwer unpublished data). Plants were subjected for 2 weeks to the treatments. The experiment was conducted in a heated greenhouse. Incident light was supplemented by high pressure sodium lamps (Hortilux Schreder, 600 Watt) and was on average 297 ± 13 (1se) µmol m^{-2} s^{-1} during the experiment.

Measurements

All measurements were performed on the third youngest ramet with a fully unfolded leaf lamina. As successive ramets can differ in their petiole length dependent on their developmental stage and the developmental speed can differ among treatments, we measured petioles of the same developmental stage (i.e. a local plastochron index of 3 (Birch and Hutchings 1992; Huber and Stuefer 1997)).

The third-youngest petiole was detached with a razor blade at its base, and its length and diameter in two perpendicular directions were measured with a caliper to the nearest millimeter and a leaf thickness meter to the nearest 0.01 mm, respectively. Leaf lamina area was measured with a leaf area meter (Licor, LI 3100). The petiole diameter measured perpendicular to the surface of the leaf lamina was used for further calculations.

Young's elasticity modulus (E, MN m^{-2}, which is a measure for the rigidity of a material, was measured with a universal material testing machine (Instron Model 5542, Canton, USA) using a three-point bending method following Liu et al. (2007). This method has the advantage that it keeps the force perpendicular to the petiole. The middle section of the petiole was placed horizontally over two supports that were 2–3 cm apart. The distance was adjusted such that it was two-thirds of the length of the petiole segment. Vertical applied forces (F, N) and resulting deflections (δ, m) were recorded. Young's modulus was calculated as follows (Gere and Timoshenko 1999):

$$E = (FL^3)/48\delta I \tag{1}$$

where L is the length between the supports (m) and I the second moment of area (m^4), which is a measure for the degree to which the cross sectional area of a support member contributes to mechanical stability (Gere and Timoshenko 1999). I was calculated from the cross-sectional dimensions of the petiole assuming it to have a parabolic shape (see Fig. 3.3. in (Niklas 1992)):

$$I = (16/175)r_a^3 r_b \tag{2}$$

with a length equal to r_a and a width equal to $2*r_b$ (Niklas 1992). Also the flexural stiffness of the petiole was calculated as the product of E and I (EI, MN m^2).

Immediately after measuring biomechanical characteristics, we made epidermal imprints of each petiole. This was done by gently placing the adaxial side of the petioles on liquid rubber (Coltende President jet Plus, Altstatten, Switzerland). This rubber hardens within 2–3 min, after which the petiole can be removed. The dried rubber contains an imprint of the epidermal layer of the whole petiole. This imprint was used as a mould and prints of the moulds were made with clear nail polish. From these prints total cell number and cell length can be estimated (Ridge and Amarasinghe 1984). Once dried, the prints were carefully removed from the moulds and put on a cover glass. These prints showed clear patterns of the upper layer of the petiole under a light microscope (magnification = 200). Three different randomly chosen places were used to determine the cell number per millimeter. Areas around stomata and directly adjacent cells were not measured because these cells have markedly different sizes.

Leaf and petiole dry mass was determined after drying leaves and petioles to constant weight at 72° for 48 h.

Statistical analyses

A mixed model ANOVA (SAS Procedures PROC GLM) was used to test for the effects of treatment and genotype on plant traits, with treatment and temporal blocks (see Treatments) being treated as fixed effects and genotypes as random effects. The effects of mean trait value and treatment on mean Young's modulus and mean flexural stiffness were tested with an ANCOVA using within treatment genotypic means. In this analysis a significant effect of traits indicates that in addition to the overall treatment effects, developmental and phenotypic plant traits, which were introduced as covariates into the model, affect the biomechanical characteristics. Genotypic correlations among all traits were calculated for each treatment separately.

To test for the direct and indirect effects of developmental and phenotypic traits on biomechanical characteristics, we performed a path analysis using the program package AMOS (Arbuckle and Wothke 1999). Path analytical models can be used to explore and quantify patterns of variation in character correlations (Pigliucci and Kolodynska 2006) Cell number, cell size, petiole diameter and leaf area were entered in the program as exogenous traits and correlation coefficients among those traits were calculated. Petiole length, leaf weight, Young's modulus and flexural stiffness (EI) were entered as endogenous traits. We tested the effects of the exogenous traits on the endogenous traits as well as the interrelationships between cell number, petiole length, and leaf area and the effects of these traits on biomechanical characteristics. Further we calculated the paths of petiole diameter and elasticity modulus on flexural stiffness of the petioles. This analysis allows for testing which traits exert direct effects on flexural stiffness and which traits affect flexural stiffness via modification of the petiole diameter and the material properties of the petioles (Young's modulus), respectively. For this analysis all plants subjected to a common treatment, and not genetic means, were used.

In order to test for the effects of traits on performance we regressed traits on performance, using the performance data on the same genotypes published in (Weijschede et al. 2006), assuming that the traits measured in the present experiment represents a stable trait characterizing the respective genotypes across experiments and can therefore be used to assess the underlying mechanisms explaining variation in performance across experiments. In the experiment of Wijschede et al. (2006) the same genotypes were grown under high light conditions as well as under vertical light gradient and homogeneous shade (Weijschede et al. 2006). We used the performance data (i.e. ramet production) for the control conditions and the plants subjected to homogeneous shade, as these treatments were comparable to the treatments employed in the present experiment. Petiole length was added to the analysis to account for differences in performance inherently correlated to petiole length expression (Weijschede et al. 2006), enabling us to distinguish the effects of petiole length and the traits of interest. From that analysis one can infer in how far the respective traits affect plant performance, whether the effects differs among genotypes expressing different petiole length and the direction of the response.

Results

Individual leaf traits

Plants grown under shaded conditions produced significantly longer petioles which tended to be slightly thinner (Fig. 1, Table 1). Shading significantly increased allocation to

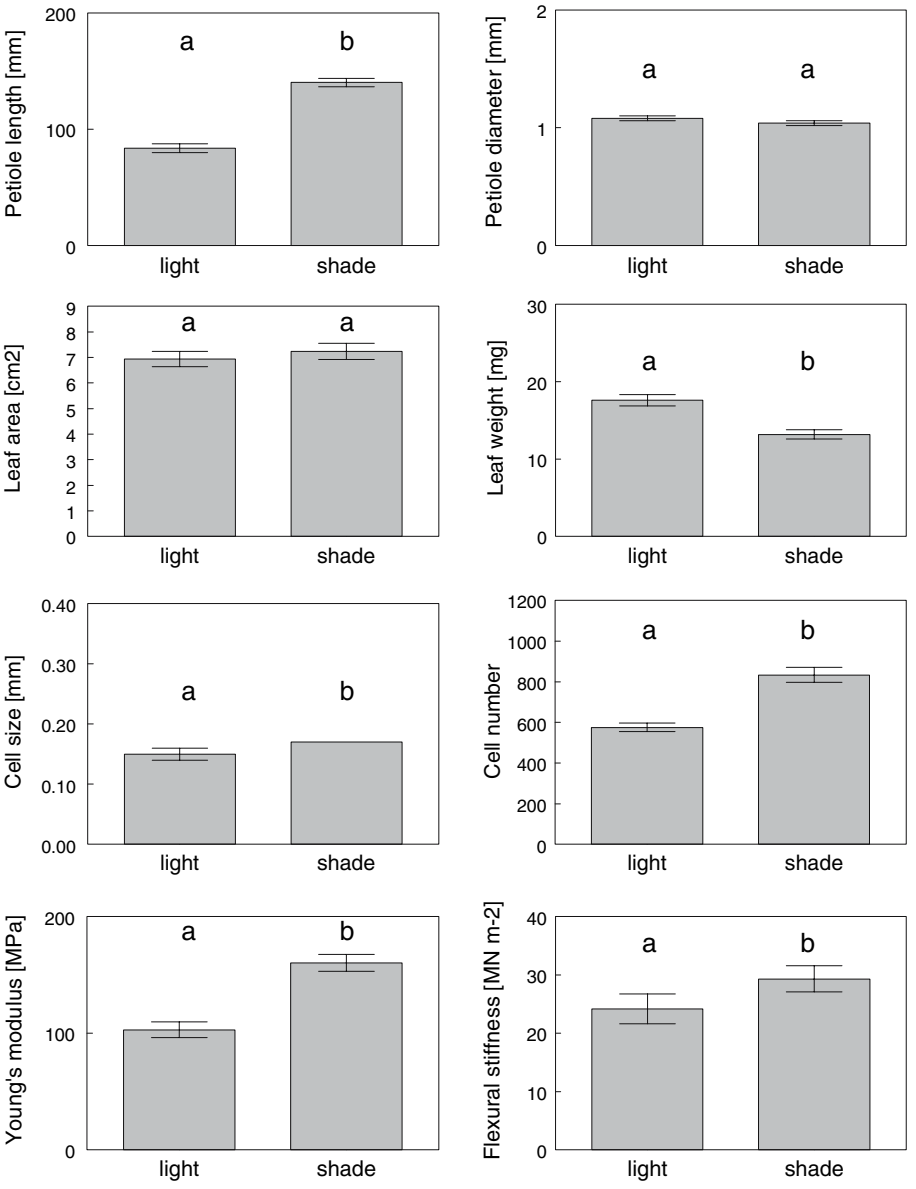

Fig. 1 Mean (±1SE) effect of the two treatments on morphological, developmental and biomechanical plant traits. Different letters indicate significant differences between treatments at $P \leq 0.05$

petioles. On average individual ramets (internodes, leaves and petioles) of shaded plants invested 42% of their biomass into petioles, while plants grown under high light conditions allocated 27% of their weight into petioles. If calculated per leaf, shaded leaves allocated 49%, and leaves grown under high light conditions allocated 35% of their biomass into petioles. Lamina area of individual leaves was the same in the two shading treatments, but leaf mass (laminas and petioles together) was negatively affected by shading. The

Table 1 Mixed model ANOVA (SAS Procedure PROC GLM) results on the effects of light treatments and genotypes on morphological, developmental and biomechanical plant traits

	Treatment	Genotype	Treatment*genotype	Block
df	1	13	13	3
Petiole length	180.59***	4.44***	1.52 ns	28.13***
Petiole diameter	3.29$	8.61***	1.88*	6.24***
Leaf area	1.04 ns	17.73***	2.06*	14.27***
Leaf weight	9.88***	12.61 ***	0.90 ns	4.26**
Cell size	30.52***	2.76**	0.68 ns	6.74***
Cell number	47.06***	4.88***	1.22 ns	9.05 ***
Young's modulus	95.34***	0.96 ns	0.31 ns	1.24 ns
Flexural stiffness	5.56*	0.07***	0.72 ns	7.91***

The F-values and their significances are given. Significance levels are: ns: $P > 0.1$; $ $0.1 > P > 0.05$; *$0.05 > P > 0.01$, **$0.01 > P > 0.001$; ***$P > 0.001$

epidermis of shaded petioles contained more cells, when counted along the petiole length, than that of light-grown plants. Individual epidermis cells were on average 50% longer. The Young's modulus and the EI of shaded petioles were higher than that of the high-light ones, indicating that for a given length, the petioles were more resistant to bending. There was a high genetic variation among the 14 genotypes for all traits except Young's modulus (Table 1). In addition, the diameter of petioles and leaf area responded significantly different to shading treatments among genotypes.

Genotypic trait correlations revealed that cell number was significantly positively correlated with petiole length, petiole diameter, leaf area, and leaf weight in both light conditions (Table 2). Under shaded conditions cell size was negatively correlated with area and weight of leaves, as well as with cell number. These correlations were not significant under high light conditions.

This study shows the absence of genetic correlations between the Young's modulus with any of the other measured plant traits in both light conditions (Table 3). However, there was a consistent positive correlation between flexural stiffness and other plant traits. Under both light conditions genotypes producing petioles with greater flexural stiffness were also characterized by longer and thicker petioles, larger and heavier leaves and the petioles consisted of more cells. Under low light conditions, but not under high light conditions, there was a negative correlation between flexural stiffness and cell size.

Influence of leaf traits on biomechanical characteristics

The Young's modulus was mainly affected by shading, while the flexural stiffness was, in addition to treatment effects, also affected by other developmental and morphological traits (Table 3). Flexural stiffness was affected by petiole length, petiole diameter, leaf area, leaf weight, cell number and cell size with the rigidity of petioles decreasing with increasing cell size and increasing with an increase of the other traits.

The data reveal significant correlations between cell number and cell length and the flexural rigidity of the petioles, but not with their tissue properties (Table 2). However, the strength of the correlation between cell size and cell number differed between treatments. Under high light conditions cell number was positively correlated with petiole diameter,

Table 2 Genotypic correlation among morphological, developmental and biomechanic traits

		High light							
		Petiole length	Petiole diameter	Leaf area	Leaf weight	Cell number	Cell size	Young's modulus	Flexural stiffness
Low light	Petiole length	.	0.85***	0.75**	0.68**	0.75**	0.17 ns	0.07 ns	0.85*
	Petiole diameter	0.78**	.	0.63*	0.61*	0.67**	0.25 ns	–0.14ns	0.91***
	Leaf area	0.56*	0.81***	.	0.98***	0.98***	–0.41 ns	0.23 ns	0.81***
	Leaf weight	0.65*	0.91***	0.96***	.	0.83***	–0.44 ns	0.24 ns	0.79***
	Cell number	0.80***	0.70**	0.68**	0.69**	.	–0.42 ns	0.06 ns	0.76**
	Cell size	–0.45 ns	–0.45 ns	–0.61*	–0.05*	–0.86***	.	0.15 ns	–0.12 ns
	Young's modulus	–0.06 ns	–0.17 ns	–0.06 ns	–0.09 ns	–0.05 ns	0.11 ns	.	0.48 $
	Flexural stiffness	0.70**	0.94***	0.77 **	0.77**	0.73***	–0.55*	0.35ns	.

Correlations were calculated for each treatment separately using the genotypic means ($n = 14$). Correlation coefficients above the diagonal indicate correlations expressed under high light conditions, Correlation coefficients below the diagonal indicate genotypic correlations expressed under low light conditions. For significance levels see Table 1

Table 3 ANCOVA testing for the effects of treatment and plant traits expressed by a genotype on the average biomechanical characteristics expressed by a genotype

	Young's modulus			Flexural stiffness		
	r^2	Treatment	Trait	r^2	Treatment	Trait
Petiole length	0.64	9.46**	0.00 ns	0.57	18.08***	31.92***
Pet. diameter	0.65	41.23***	0.61 ns	0.87	27.35***	155.1***
Leaf area	0.64	43.75***	0.14 ns	0.64	1.28 ns	42.11***
Leaf weight	0.64	36.53***	0.18 ns	0.62	19.38***	37.45***
Cell size	0.64	31.29***	0.01 ns	0.16	3.92$	3.65$
Cell number	0.64	25.01***	0.98 ns	0.56	5.50*	29.63***

Please note that this analysis has been done on the genotypic means calculated within treatments. For significance levels see Table 1

thereby also affecting flexural stiffness, indicating that petioles constructed of more cells were thicker and by consequence more resistant to bending. Under shaded conditions this correlation was maintained. In addition cell size was negatively correlated with both diameter and flexural stiffness, indicating that petioles constructed of larger cells tended to be more flexible.

There were significant correlations between shade induced changes of cell number and cell size and shade induced changes in the Young's modulus (Fig. 2). Genotypes increasing their number of cells in response to shading also experienced a relative increase in the tissues rigidity. Shade induced increase in the size of the cells, on the other hand,

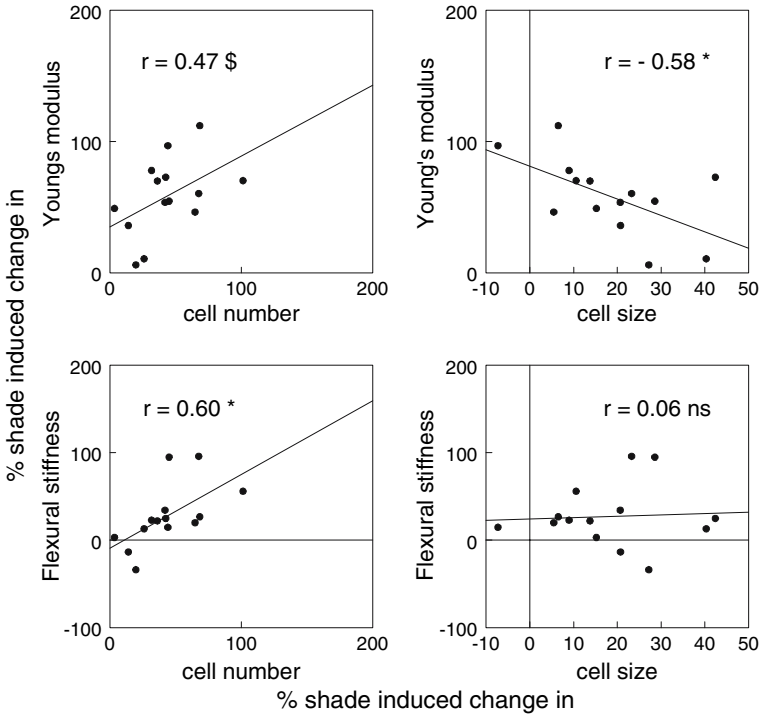

Fig. 2 Effects of shade induced change in cell size and cell number on shade induced change in Young's modulus and flexural stiffness. In the graph the correlation coefficient (r) and its significance is given. Significance levels are as in Table 1

leads to decreased tissue rigidity. Shade induced increase of cell number leads also to an increased flexural stiffness of the petioles, whereas shade induced increase of cell size did not affect shade induced changes in the flexural stiffness (Fig. 2).

The phenotypic path analysis revealed complex inter-relationships among traits (Fig. 3). The strength and direction of these relationships were affected by the light environment. Independent of light conditions cell size and cell number were negatively correlated and an increase in both lead to the production of longer petioles; though the effect of cell number was greater. Light availability distinctively altered the pattern and direction of the effects of cell number, cell size and petiole length on biomechanical characteristics of the petioles. While under high light condition increased petiole length was associated with a reduction in the Young' modulus (E: the rigidity of petiole tissue) and to an increased flexural stiffness of the whole petiole; the increase in diameter and associated second moment of area (I) more than compensated for the effect of a lower E. Under shaded conditions petiole elongation tended to have a positive effect on E and no direct effect on the flexural stiffness. Under both conditions the exogenous variables cell number, petiole diameter and leaf area were positively correlated with each other. Leaf area had a consistent indirect positive effect on flexural stiffness by way of leaf area being positively associated with leaf weight, which in turn positively affected the elasticity modulus. In contrast to high light conditions, where leaf area also directly positively affected the elasticity modulus, this effect was negative under shaded conditions.

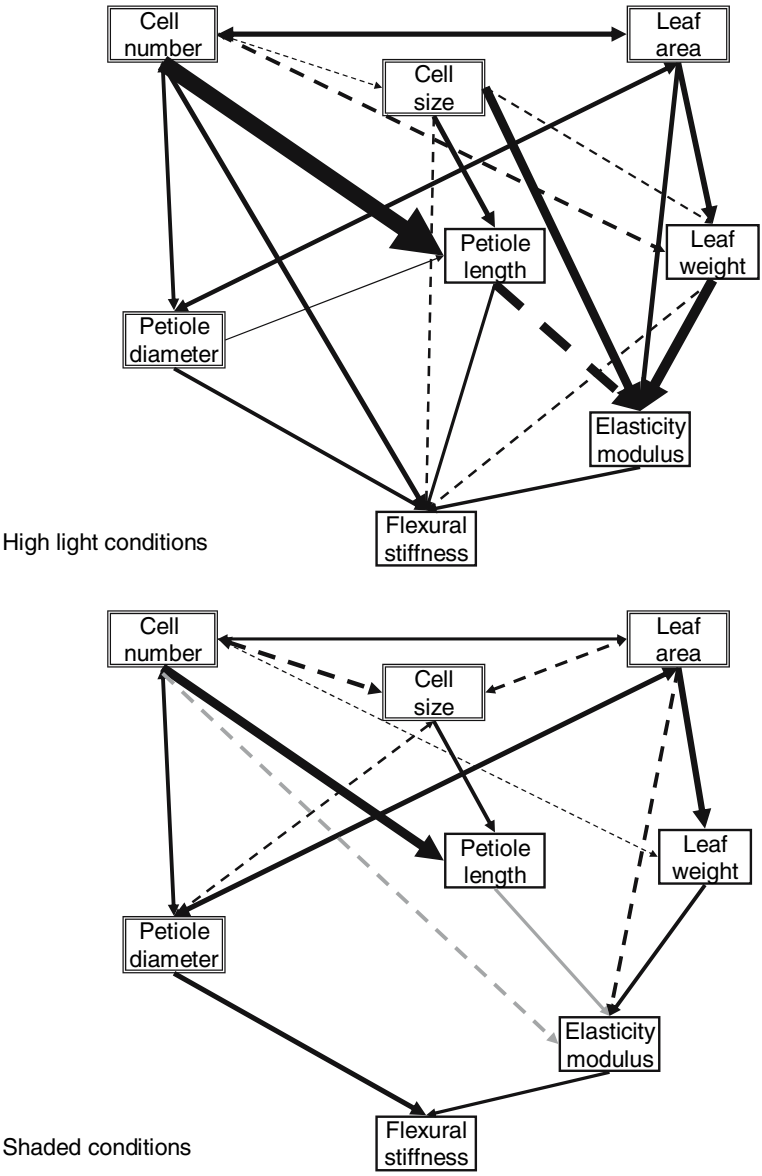

Fig. 3 Results of a phenotypic path analysis depicturing the underlying relationships among morphological and developmental plant traits and their consequence for biomechanical characteristics. Cell number, cell size, diameter and leaf area were assumed to be exogenous traits and are placed in a double lined box, the other endogenous traits are placed in single lined boxes. Correlations among exogenous traits were calculated, indicated by double headed arrows. The thickness of the lines indicates strength of the effects. Black lines indicate significant effects, grey lines non-significant effects with the standardized estimate exceeding 0.5. Non significant paths with a standardized estimate below 0.5 are not represented. Full lines depict positive relationships, dashed lines negative relationships. The analysis was done for plants subjected to high and low light conditions separately

Table 4 Effects of morphological and biomechanical characteristics on plant performance (measured as ramet production) under shaded conditions

	r^2	Petiole length	Trait	Length*trait
Petiole diameter	0.49	2.14$	2.62*	–2.37*
Leaf area	0.62	2.15$	3.40**	–2.85*
Leaf weight	0.57	0.01$	3.08*	–0.02*
Cell size	0.55	–3.08*	–3.03*	2.92*
Cell number	0.73	3.95**	4.68***	–4.50**
Young's modulus	0.22	0.30 ns	–0.05 ns	–0.44 ns
Flexural stiffness	0.64	1.65 ns	3.66**	–3.1*

Petiole length is added to the analysis as well to take account for the pure effects of petiole length on plant performance. Analyses are done on the genotypic means of plants grown in homogeneous shade. T-values and their significances are given. For significance levels see Table 1

Influence of leaf traits on plant performance

The diameter of petioles, the cell number per petiole and the flexural stiffness, but not the Young's modulus was significantly correlated with ramet production of plants subjected to shade (Table 4). There was no effect of leaf traits on performance of plants under high light conditions (data not shown). Plants which produced thicker petioles produced on average significantly more ramets if grown under shaded conditions than plants with thinner petioles. There was also a significant negative interaction between petiole length and petiole diameter on plant performance Also petioles containing more cells produced on average more ramets. Comparable to the effects of petiole thickness, there was a significant negative interaction of petiole length and cell number on plant performance. Leaf area and leaf weight followed the same qualitative pattern as petiole diameter and cell number. Overall these results indicate that producing taller more massive leaves, thicker petioles and investing into cell division positively affected plant performance under shaded conditions. Also higher flexural stiffness had a positive effect on plant performance. Genotypes producing stiffer petioles under shaded conditions produced more ramets. This indicates that there are no costs associated to the production of stiffer petioles. However, we also found a significant negative interaction between petiole length and flexural stiffness, indicating that for longer petioles it was favorable to be less stiff, whereas for shorter petioles it was more favorable to be stiffer. There was no effect of the Young's modulus on plant performance.

Discussion

Shade avoidance is very common in many plant species (Morgan and Smith 1979; Schmitt et al. 2003; Schmitt and Wulff 1993; Sultan and Bazzaz 1993; Weinig 2000). In stoloniferous plants adaptive plasticity to shading is achieved by the production of longer petioles that reach higher positions in the canopy (Huber et al. 1998; Huber and Wiggerman 1997; Leeflang et al. 1998). Beyond this obvious response, shade induced elongation processes entail a multitude of other structural and developmental changes (Cipollini and Schultz 1999; Maliakal et al. 1999; Schmitt and Wulff 1993; Smith 1982). While the benefits of shade induced elongation responses are beyond doubt, the consequences of the structural

changes associated with these responses are still under investigation. In this paper we show how structural and developmental changes in concert result in the production of more rigid petioles. A better understanding of the effects of structural and developmental changes associated with shade induced elongation responses on biomechanical characteristics and ultimately on plant performance will enhance our understanding of the evolution of shade induced elongation responses in stoloniferous plants. It may also shed light on whether evolutionary trajectories are different for shade induced elongation in vertical spacers of clonal and non-clonal plants.

Biomechanical properties affected by light conditions

In general shaded petioles had a higher Young's modulus (E) than unshaded ones, which is consistent with previous findings for both stems (Anten et al. 2005) and petioles (Niinemets and Fleck 2002; Liu et al. 2007). This result could be attributed to a greater turgidity of stem tissue which tends to be greater in shade grown plants (Liu et al. 2007; Niklas and Owens 1989). The stiffness of herbaceous support structures is largely the result of the rigid epidermis and possibly one or two underlying cell layers being held in tension by a hydrostatically inflated inner core (Hofmeister 1859; Niklas and Paolillo 1997). Thus tissue rigidity (E) in such structures depends not only on tissue composition but also on cell turgor (Niklas 1989; Niklas et al. 1999). Direct measurements have shown strong positive correlations between E and stem water potential or water content (Niklas 1989; Niklas and Paolillo 1997). For giant petioles of *Amorphophallus titatum* growing up to several meters in height, a clear positive correlation between turgor pressure and E was also found (Hejnowicz and Barthlott 2005). Shade induces stem elongation but simultaneously suppresses photosynthesis and thus assimilate supply for growth. An increased turgor pressure may then be an energy efficient way of obtaining the rigidity necessary for self support (Liu et al. 2007). However this mechanism of increased turgor will not change the modulus of rupture, which depends largely on the material properties of cell walls (Niklas 1994). Thus an increased rigidity (i.e. reduced flexibility) makes petioles more vulnerable to failure under external forces such as wind loading or trampling (Ennos 1997).

Light environment and plant traits interact in determining Young's modulus and flexural stiffness

The flexural stiffness of a petiole depends both, on the cross sectional area and on the material property of the tissue it is constructed of (Niklas 1992). Our results show that the mechanical tissue properties are mainly affected by the light environment, as petioles produced under low light conditions consist of more rigid tissue than petioles produced under high light conditions. Leaf area, leaf weight and petiole length interact in affecting the material properties. The direction of the effects of petiole length and leaf area on the material property were, however, distinctly different between light treatments, which may also explain why we did not detect general effects of morphological and developmental traits on tissue rigidity. These results provide evidence that shade induced plasticity of phenotypic traits can alter inter trait correlations (Malausa et al. 2005; Stanton et al. 2004) and that the traits, though interrelated in high light conditions do not respond to shading in concert and that trait correlations may be broken up under resource poor conditions.

A similar, but even more extreme pattern emerged for flexural stiffness, which also increased in shaded plants. While flexural stiffness of the petiole was directly affected by various morphological traits under high light conditions, these effects were, if they were present at all, only indirect in low light conditions. Only petiole diameter, which did not respond to shading, and the Young's elastic modulus, which was increased under shaded conditions directly affected the flexural stiffness of petioles under low light conditions. The strength of correlation was thus generally weaker under low light than under high light conditions, which is in contrast with the notion that the pattern and strength of integration among parameters is stronger in plants experiencing low resource status (Cheplick 2001; Huber et al. 2004) and other studies that have found that the pattern of phenotypic integration changes little between treatments (Pigliucci and Kolodynska 2002, 2006). In concert these results indicate that the tissue rigidity and the flexural stiffness of the petiole may be fine-tuned depending on other morphological characteristics such as a given length of a petiole, the leaf area a petiole has to support or the weight of the leaf lamina. However, the direction and magnitude of effects differs between light environments. The generally smaller effects of other correlated phenotypic traits on biomechanical characteristics may indicate that shaded conditions lead to a stronger canalization of the expression of mechanical properties.

Surprisingly petioles were hardly thinner under shaded conditions, which is in contrast to the findings for stems of erect plants. In stems of erect plants internode thickness can be modified throughout ontogenetic development by means of secondary growth (Esau 1977). If mechanical stability of stem internodes proves not to be high enough to accommodate increasing strain on the stems caused by the acropetal addition of new modules, flexural stiffness of the stems can thus still be adjusted. This continuous ability of internodes to adjust their thickness may enable erect plants to initially invest resources economically into height growth and the production of new modules instead of increased stem thickness. In petioles secondary growth is much less common (Esau 1977). As soon as petiole length growth and lamina expansion have finished only external forces, but not internally increased biomass load, may exert extra force on the petioles. The limited possibility of secondary growth may necessitate petioles to be constructed of sufficient strength to withstand unpredictable external forces and does thus not allow for the economic production of initially thinner petioles.

Effects of cell size and number on biomechanical properties

Read and Stokes (2006) have argued that fundamental design traits at both the cellular and whole plant level are directly influenced by the immediate environment. Structure, size and alignment of epidermal cells have been argued to affect biomechanical tissue properties (Loodts et al. 2006). To the best of our knowledge the effects of genotypic variation in cell size and cell number on the material properties of petioles and the resulting flexural stiffness have not been investigated previously. Our data clearly show that biomechanical characteristics of the petioles depend on the developmental mechanisms controlling petiole length. Under high light conditions petioles containing more cells had a higher flexural stiffness. This was achieved both by a direct effect of cell number on petiole diameter and associated second moment of area, and indirectly by a positive effect of cell number on the petiole length, which in turn positively affected flexural stiffness. In *Trifolium repens* traits such as lamina size, petiole length, internode length and petiole thickness are strongly correlated (Weijschede et al. 2006). This may indicate that the same developmental

process, i.e. magnitude and speed of cell proliferation, is responsible for within treatment variation in petiole length and thickness, and ultimately for flexural stiffness. However, we did not measure the horizontal extension of cells, and can thus not prove this hypothesis.

Contrary to our expectations, between genotypes, there was no negative correlation between the size of epidermal cells and the Young's modulus (E) of the petiole. One explanation could be that differences in other petiole characteristics masked the effect of cell size on E. First, E is largely determined by the turgor pressure exerted by the inner core of the petiole, which in turn is regulated by the maintenance of osmolarity within cells (Liu et al. 2007). Second, the genotypes probably differed with respect to tissue characteristics other than the length of epidermal cells, such as the cell wall characteristics of epidermis cells and the relative amount of collenchyma, which may also influence E (Niklas 1994).

On the other hand, in accordance with our prediction, plastic shade induced elongation of cells was negatively correlated with the shade induced increases in E (Fig. 2). This suggests that increased cell elongation may indeed negatively impact E. We also observed that while increased cell length in the shade was associated with a greater petiole length in the shade, it was not correlated with a concomitant increase flexural stiffness (EI, Figs. 2 and 3). Without a change in EI a taller structure is more likely to buckle and thus our notion that while cell elongation might be an energy efficient way of increasing petiole length as compared to cell division, it can result in lower mechanical stability seems to be supported.

Costs and benefits associated to shade induced elongation responses

Shade induced elongation of spacers has been hypothesized to be associated to costs in terms of decreased biomechanical stability, which may increase the risk of lodging or breaking (Anten et al. 2005; Dudley and Schmitt 1996; Huber et al. 1998; Huber et al. 2004). Under shaded conditions the relative amount of carbohydrates allocated to elongating petioles is strongly increased in *T. repens*. In comparison to a wide range of species (Niinemets et al. 2006) the relative allocation to elongated petioles positioning and supporting the leaf laminas is relatively high and surpassed by only very few species. This indicates that shade induced elongation processes are associated to high costs in terms of biomass investment into support tissues. However, even increased resource allocation to the elongating organ may not be sufficient to match the increased resource demand, resulting in thinner and weaker stem internodes or petioles. Biomechanical needs can be matched by either changing tissue properties or reallocating tissue in a more efficient way (Niklas 1992). Our data provide evidence that cell number and flexural stiffness, but not tissue rigidity confer a selective advantage under shaded conditions. Increased cell size, on the other hand, was associated with decreased ramet production. Interestingly, the same traits did not affect performance under high light conditions. These data show that increasing the number of cells, decreasing the size of cells, or increasing the flexural stiffness is not associated to costs, even in an environment providing homogeneous shade, where the selection pressures are supposed to be lower as lodging will not result in decreased light interception. This indicates that the increased structural demands necessary for producing smaller cells (i.e. higher number of cell walls) or for producing thicker, and thus stiffer petioles, may not confer costs and lead to reduced plant performance. Plastic or constitutively increased flexural stiffness and the production of more and smaller cells will be selected for in shaded environment and constitutively higher values for those traits will not be selected against under unshaded conditions.

A broader range of genotypes has shown that both strategies, elongating petioles by means of increased cell number and cell size (Weijschede et al. submitted) are maintained in a population, supporting the notion that the benefits associated to the production of stiffer petioles may outweigh any structural costs potentially incurred under natural conditions. The net benefits associated with increased petiole stiffness differed among light environments. Small scale temporal and spatial heterogeneity may lead to the maintenance of different investment strategies into petiole rigidity under natural conditions. As stolons of stoloniferous plants spread horizontally throughout the vegetation, even successive ramets on an integrated clonal system may experience different selection regimes, and may thus experience advantage, as well as disadvantage of investing into increased cell number or size and the associated costs and benefits.

Conclusions

In short, changes in Young's modulus in response to shading were negatively correlated with changes in cell size while shade induced changes in cell number were positively correlated to changes in Young's modulus and flexural stiffness. A large flexural stiffness in turn was associated to increased fitness in plants under shade but not under high light conditions, which indicates that the pattern of selection on flexural stiffness, and thereby also on shade induced changes in cell size and cell number, differs among light environments.

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